

Enzyme Inhibitors

Protein-tyrosine kinase inhibitors

Terrence R. Burke, Jr.

Laboratory of Medicinal Chemistry, Developmental Therapeutics
Program, Division of Cancer Treatment, National Cancer
Institute, Bethesda, MD 20892, USA

CONTENTS

Introduction	119
Inhibitors of nucleotide binding	120
Inhibitors of phosphoacceptor interaction at the catalytic site	122
Bisubstrate analogs as inhibitors at the catalytic site	124
Inhibitors of substrate recognition outside the catalytic site	126
Agents which inhibit by other mechanisms	126
Conclusions	128
Addendum	128
Acknowledgements	128
References	128

Introduction

Protein-tyrosine kinases (PTKs) constitute a class of enzymes which catalyze the transfer of the γ -phosphate of either ATP or GTP to specific tyrosine residues in certain protein substrates. Evidence suggests that these enzymes are important mediators of normal cellular signal transduction (1), with PTKs being the intracellular effectors for many growth hormone receptors (2-4). PTKs are also frequently the products of proto-oncogenes and their aberrant expression has been associated with a variety of human cancers (5-7). Since the discovery a little over 10 years ago that the transforming gene product of the Rous sarcoma virus is a protein kinase which phosphorylates tyrosine residues, over 40 vertebrate PTKs have been identified. These may be categorized into two main groups depending on whether they possess (receptor PTKs) or lack (non-receptor PTKs) extracellular ligand binding domains. Both receptor (2-3) and non-receptor (1) PTKs have been further classified into families based on structural homologies. The importance of PTKs in signal transduction and the association of aberrant PTK expression with proliferative disorders makes agents which modulate the activity of PTKs attractive therapeutic targets and pharmacological probes (8, 9).

An active cytoplasmic catalytic domain has been demonstrated to be essential for the ability of normal PTKs to induce mitogenesis and for oncogenic PTKs to induce and maintain the transformed phenotype (3, 10). Initial activa-

tion of kinase function is frequently associated with "autophosphorylation" (self-phosphorylation) of one or more tyrosine residues. This autophosphorylation is believed to be an intermolecular process between neighboring PTKs following oligomerization in response to some signalling event, such as growth factor binding at the extracellular ligand-binding domain of receptor PTKs (11). The mechanism by which autophosphorylation activates kinase action is not known, but may involve conformational alterations which allow substrate access to the catalytic site and the generation of secondary recognition features necessary for substrate binding (12, 13). Autophosphorylation represents one means through which general modulation of PTK function is achieved (14). Accordingly, an important feature of many PTKs is the presence of conserved, non-catalytic domains termed "SH2" and "SH3" regions, which are located both within modulatory regions of PTKs and in certain endogenous substrates (15). These regions form tight associations with specific peptide sequences containing phosphorylated tyrosine residues and appear to direct protein-protein interactions, such as binding of substrate to the PTK. Mutations in the SH2 regions have been shown to affect the ability of PTKs to associate with specific endogenous substrates and to activate transforming potential (16, 17). Aberrations in either the ability to respond to appropriate signalling or in the discrimination of substrate can potentially lead to errant signal transduction and neoplastic proliferation.

It is possible to delineate several common motifs which can serve as a basis for the design of PTK inhibitors (18-20). Central to the function of all PTKs is the recognition and binding of a nucleoside triphosphate (usually ATP) and an appropriate tyrosyl-containing substrate, followed by the ensuing direct transfer of phosphate between the two (21, 22). (Note: Although ATP and tyrosyl-containing peptide are technically both substrates, for purposes of this review the term "substrate" will be used to designate the latter only). In considering agents which inhibit PTK function, particular emphasis will be placed on those compounds which appear to act by interfering with the catalytic process itself. Agents which inhibit this process can conceptually be classified as 1) preventing the binding of ATP; 2) preventing the binding of substrate; 3) decreasing the catalytic efficiency of the enzyme by some other mechanism. Agents which prevent the binding of substrate can be further delineated as those

which prevent the binding of the tyrosyl residue within the catalytic site and those which inhibit the recognition and association of the PTK with the larger substrate (for example, agents directed against SH2-interactions).

Inhibitors of nucleotide binding

An X-ray crystal structure of the catalytic subunit of a cyclic AMP-dependent protein kinase has recently been reported (23). Although this enzyme is a serine/threonine kinase, it affords insight into the catalytic domain of PTKs due to the high degree of homology among the catalytic domains of all protein kinases (24). It was observed in this case that the adenine ring of ATP is situated deep within a large cleft beneath a beta-sheet, the ribose sugar and triphosphate chain of ATP being more proximal to the cleft opening where actual catalysis presumably occurs. A similar architecture may be involved in the binding of ATP to PTKs.

A variety of compounds have been shown to inhibit the function of PTKs in a manner which is competitive with respect to nucleotide binding. The fact that these agents usually contain a planar portion, multiple aromatic rings and a variety of heteroatoms whose nature and location are extremely important for activity, is consistent with interaction at a cleft site which also recognizes the planar, highly functionalized adenine ring of ATP. However, the absence of 3-dimensional structural information correlating molecular features of these inhibitors with ATP has impeded the development of new analogs based on rational mechanism-based approaches. Since a high degree of homology exists among the nucleotide binding domains of many ATP-utilizing enzymes, problems with a lack of specificity are potential side effects of agents which act at the ATP binding site. Increasing kinase specificity is, accordingly, one important focus of research in the development of such compounds.

Nucleoside-based inhibitors

Nucleoside-based analogs were among the first agents explored as potential inhibitors of ATP binding to PTKs. The known serine/threonine kinase inhibitor 5'-[4-(fluorosulfonyl)benzoyl]adenosine (5'-FSBA) **1** (Fig. 1) has been shown to form covalent bonds to lysine residues within the active site of PTKs (25). Additionally, both ADP and GDP, as well as the unusual diadenosine 5',5''-P₁P₄ tetraphosphate (Ap₄A), have also been shown to inhibit PTK activity (26). Problems with membrane transport coupled with a lack of specificity and potency make these compounds unpromising as therapeutic leads.

Flavonoid inhibitors

Flavonoids possess a variety of biological properties, including the ability to interact with nucleotide binding sites in several different enzyme systems (27). A number of naturally occurring flavones (of which quercetin **2** is an important example) and isoflavones (typified by genistein **3**) have been shown to inhibit the function of PTKs in a manner

which is competitive with respect to ATP (28). Flavones and isoflavones are isomeric heterocycles having placement of a phenyl ring at either the 2-position (flavones) or 3-position (isoflavones) of a benzopyrone ring system. These naturally occurring compounds differ principally in the distribution of hydroxyl or methoxyl groups at positions 3,5,7 of the benzopyrone ring system and positions 3' and 4' of the phenyl ring. In general, inhibitory potency is enhanced with increasing numbers of hydroxyls at these positions (28). Potency is reduced if hydroxyls are replaced by methoxyls, while a double bond between carbons 2 and 3 is required for activity (29). Flavones and isoflavones differ in their inhibitory profiles both in their relative selectivity towards PTKs versus serine/threonine kinases (30) and in their potencies among different PTKs (29). It has been suggested that these compounds exhibit multiple ways of interacting with PTKs (30). The lack of well defined QSAR correlations for PTK inhibitors in general, and for flavonoids in particular, may be a reflection of these multiple modes of interaction. The additional ability of flavonoids to exhibit superposition of critical molecular functionality in multiple orientations further complicates the rational modification of these agents (Fig. 2).

In spite of these ambiguities, analogs have been prepared which exhibit improved potency and selectivity. Some of these synthetic flavonoids contain nitrogen functionality which may afford recognition features presented by nitrogens in the adenine ring of ATP. These modifications have occurred at either the 2 (31) or 4' (Compound **4**) (32) positions and have sometimes resulted in compounds with enhanced PTK inhibitory activity (32). Additionally, a large series of flavones containing nitrogen functionality at the 8 (Compound **5**) (34) and 5,4' positions (34) exhibited good antiproliferative activities, although PTK inhibition was not measured. The fact that PTK inhibitory potency does not always correlate with antiproliferative potency (31) suggests that factors other than simple PTK inhibition may be involved in the biological activity of flavonoids in whole cell or animal systems (35).

Heterocycle-containing inhibitors

1) Amiloride

A variety of non-flavonoid heterocycles inhibit PTKs in a manner which is competitive with respect to ATP. Amiloride **6**, which bears some resemblance to adenine, is competitive with respect to ATP and has been shown to inhibit a number of PTKs at levels which approximate those required to block mitogenic response to growth factors (36). Although amiloride has been shown to inhibit tumor growth *in vivo* (37), its inhibition of a number of other kinases and its effects on multiple cellular functions diminishes its potential as a therapeutic lead.

2) Staurosporine

Staurosporine **7**, a member of the indolecarbazole group of antibiotics, was initially isolated as a fermentation product which exhibited marked cytotoxic activity. It and a number of related analogs (38, 39) have been shown to be potent,

nonspecific inhib
(38). While stu
a single class o
which is comp
other agents kn
main (40)). Thi
tency in cells is
on the intracell
therein), brings
tion of staurosp

Styryl-containi

An important
hibit PTKs by co

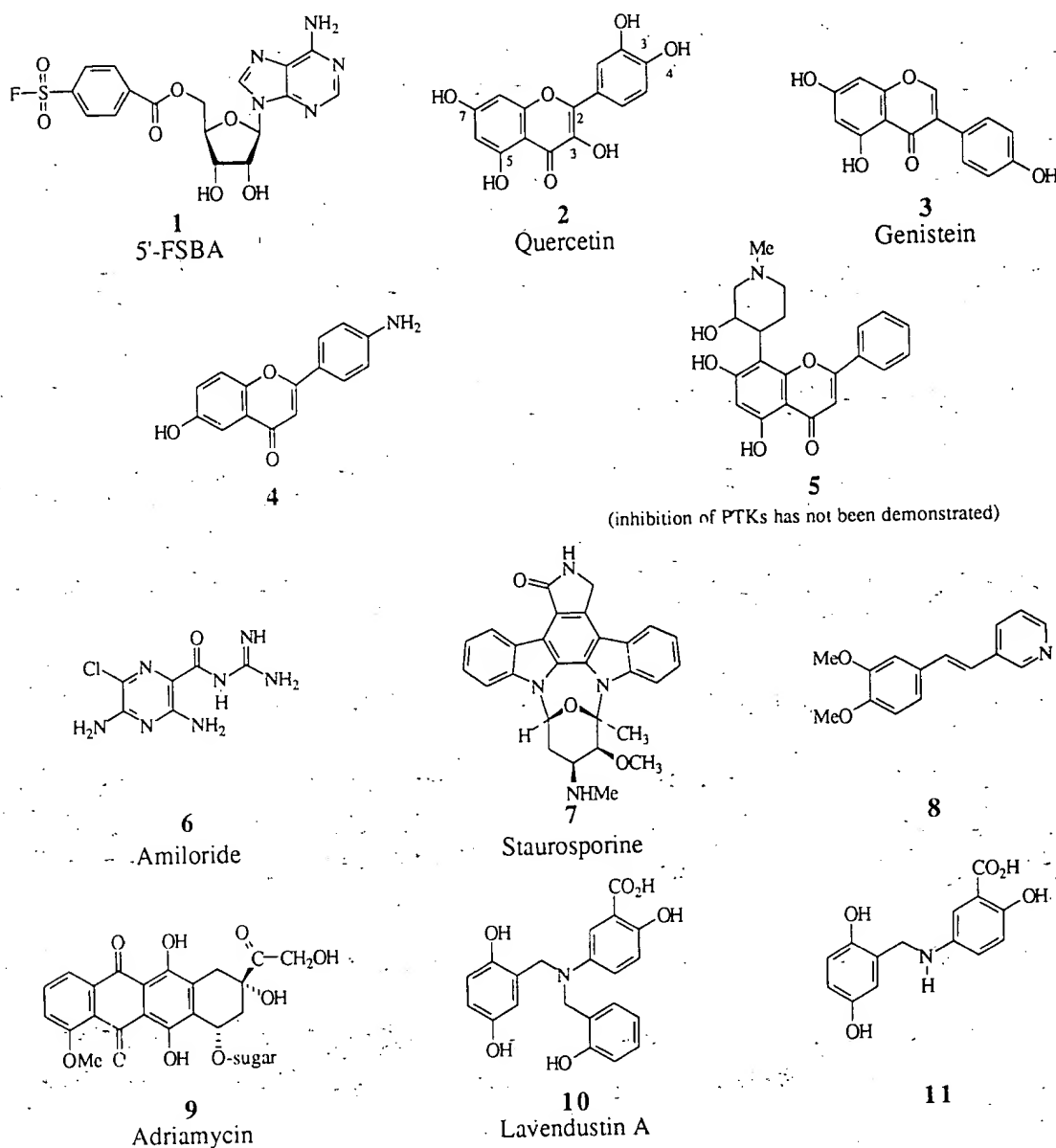


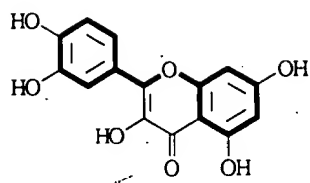
Fig. 1. Inhibitors which are competitive with respect to ATP.

nonspecific inhibitors of multiple kinases, including PTKs (38). While studies have shown that staurosporine binds to a single class of binding site in these receptors in a manner which is competitive with ATP, its binding is not affected by other agents known to compete with ATP at the catalytic domain (40)). This, when considered with the fact that its potency in cells is much greater than would be expected based on the intracellular concentration of ATP (38 and references therein), brings into question the exact nature of the interaction of staurosporine at the catalytic site.

Styryl-containing inhibitors

An important lead in the development of agents which inhibit PTKs by competing for ATP binding has been provided

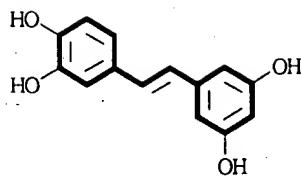
by the antileukemic compound piceatannol 19, a polyhydroxylated stilbene. While piceatannol bears striking resemblance to the flavone quercetin 2, with nearly exact superposition of both aryl ring and accompanying hydroxyls (Fig. 2), it has been shown to inhibit PTKs by competing with the phosphoacceptor peptide (41). Piceatannol represents a hybrid structure containing features of both the flavones/isoflavones (which are competitive with respect to ATP) and the styryl-based compounds typified by erbstatin which are competitive with respect to substrate (see below). A series of derivatives of piceatannol have been prepared. Replacement of the 3,5-dihydroxyphenyl ring with 3-pyridyl ring results in a 10-fold loss of activity. However, changing the 3,4-dihydroxyphenyl ring of this compound to a 3,5-dimethoxyphenyl ring provides an analog (compound 8) which has a potency closer to piceatannol, and which is competi-



2

Quercetin

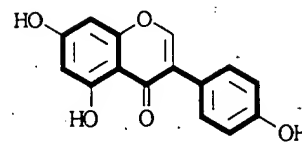
(competitive with respect to ATP)



19

Piceatannol

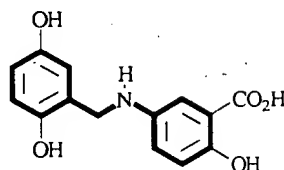
(competitive with respect to substrate)



3

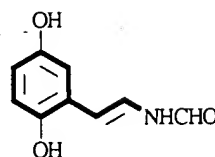
Genistein

(competitive with respect to ATP)



11

(competitive with respect to ATP)



13

Erbstatin

(competitive with respect to substrate)

Fig. 2. Common structural elements in a variety of PTK inhibitors.

tive with respect to ATP (42). This unusual alteration in the mechanism of inhibition is significant, and underscores the ability of subtle structural changes to direct agents toward either the ATP or substrate binding sites. This may provide useful information regarding enzyme-substrate and enzyme-ATP interactions.

Miscellaneous inhibitors

1) Adriamycin

Adriamycin **9** is an antineoplastic anthracycline antibiotic known for its ability to interact with DNA as well as its ability to interact with lipid membranes. Consistent with its planar, polyhydroxylated structure, adriamycin has also been shown to inhibit a number of PTKs in a manner which is competitive with respect to ATP. However, inhibition is greatly affected by the nature and concentration of substrate, indicating possible additional interactions at secondary sites (43). While adriamycin is not an effective inhibitor of many serine/threonine kinases, it does afford inhibition of protein kinase-C, limiting its value as a specific PTK inhibitor.

Lavendustin A

A potentially important lead in the development of new PTK inhibitors has been afforded by the fermentation prod-

uct lavendustin A **10**, which is an exceptionally potent competitive inhibitor of ATP binding to PTKs (44). While containing multiple aromatic rings, lavendustin A lacks the extended planarity present in many PTK inhibitors which are competitive with respect to ATP. Interestingly, the 2,5-dihydroxyphenyl group of erbstatin **13** (which is competitive with respect to substrate; see below) is also present in lavendustin A (Fig. 2) and appears to be essential for activity. Structure-activity studies have shown that simplified structure **11** retains substantial activity, and may contain key elements necessary for PTK recognition (44). Lavendustin A inhibits neoplastic cells growth in culture at concentrations significantly higher than that required to inhibit PTKs in *in vitro* assays.

Inhibitors of phosphoacceptor interaction at the catalytic site

Compounds which are competitive with respect to tyrosine-containing substrate conceptually offer a degree of selectivity towards PTKs *versus* serine/threonine kinases. However, unlike these latter protein kinases which depend on specific amino acid sequences proximal to the substrate's target residue, PTKs lack absolute recognition motifs in the vicinity of the phosphorylated tyrosine. This and the fact that very high K_m values are observed for small synthetic peptides relative to larger protein substrates, indicates that secondary or tertiary recognition features re-

moved from substrate binding site. SH3 domain (15) may provide competitive inhibition, therefore inhibiting catalytic site. Inhibition at most agents have competitive with respect to structural features of self.

Peptidic inhibitors

While not altered by PTK, peptides modeled after phosphorylation site analogs of an reasonable substrate containing tyrosine have been shown several different substituent amines with nonphosphorylated substrates has reduced the selective such replacement. An unusual analogue due has been nine, was demonstrated substrate inhibition. Extremely weakly behaved in a sine-containing phosphoacceptor site by incorporation of D-configuration. Ity than gastrin competitive with respect to affinity relative to the tyrosyl hydroxyl was suggested. It may be a transition state the ionization state phosphate. Interestingly, the L-tetrafluoroborate both ATP and p high K_m values peptide-based peptidic potential.

Amino acid-like

A series of small molecules to aromatic insulin receptor with respect to p-t-Boc-Tyr-amine

moved from the catalytic site may be responsible for substrate binding (45). As previously mentioned, SH2 and SH3 domains which reside outside of the catalytic domain (15) may participate in this recognition. Agents which are competitive with respect to phosphoacceptor substrate may therefore inhibit the interaction of the tyrosyl residue at the catalytic site, or alternatively interfere with substrate recognition at more distant "recognition domains". A number of agents have been shown by kinetic analysis to be competitive with respect to substrate and these generally contain structural features which in some manner mimic tyrosine itself.

Peptidic inhibitors

While not all tyrosine-containing peptides are phosphorylated by PTKs, a number of small tyrosine-containing peptides modeled on sequences surrounding PTK autophosphorylation sites, and several unrelated peptides, including analogs of angiotensin and gastrin, have proven to be reasonable substrates (45). Synthetic random polymers containing tyrosine, glutamic acid, alanine and lysine have also been shown to serve both as substrates and inhibitors of several different PTKs, depending on the ratios of the constitutive amino acids (46). Replacement of tyrosine residues with nonphosphorylatable residues in certain peptidic substrates has resulted in analogs which inhibit PTK function by the selective block of autophosphorylation (47); however, such replacement does not always result in inhibition (22). An unusual analog of angiotensin I, in which the tyrosyl residue has been replaced by a beta-(4-pyridyl-1-oxide)-L-alanine, was designed as a mechanism-based irreversible substrate inhibitor (48). This analog proved to be an extremely weak competitive inhibitor of substrate binding and behaved in a reversible manner. Gastrin, a small tyrosine-containing peptide which serves as a suitable phosphoacceptor substrate for many PTKs, has been modified by incorporation of tetrafluorotyrosine bearing the unnatural D-configuration. This peptide proved to have a higher affinity than gastrin and exhibited PTK inhibition which was competitive with respect to substrate (49). The increase in binding affinity relative to gastrin was attributed to ionization of the tyrosyl hydroxyl resulting from a lowering of its pKa. It was suggested that the resulting ionic species approximates a transition state analog by more closely resembling the ionization state of the phenol during the transfer of phosphate. Interestingly, the corresponding peptide incorporating L-tetrafluorotyrosine was competitive with respect to both ATP and protein substrate. Generally, because of their high K_m values, instability and poor transport properties, peptide-based inhibitors do not possess promising therapeutic potential.

Amino acid-like inhibitors

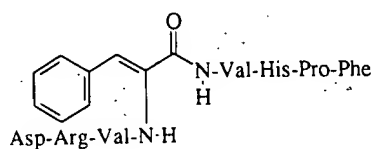
A series of small non-peptidic compounds related in structure to aromatic amino acids have been shown to inhibit the insulin receptor (IR) PTK in a manner which is competitive with respect to peptide substrate. The most effective analog, *t*-Boc-Tyr-aminomalonic acid, inhibited both exogenous

substrate phosphorylation and autophosphorylation as well as insulin-dependent lipogenesis and insulin-dependent antilipolytic effects (50). Derivatives of 3-(hydroxyphenyl)pyruvic and lactic acids have also been prepared (51). These compounds are of extremely low potency.

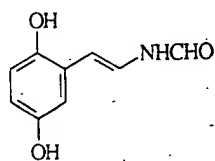
Styryl-containing inhibitors

An important structural motif which has arisen in the development of small molecules which function competitively for peptide substrate at the catalytic site was first evidenced by Wong in 1984 when replacement of the tyrosine residue of an angiotensin II analog by a dehydrophenylalanine derivative (compound 12) (Fig. 3) transformed the peptide from a PTK substrate to an inhibitor (22). The potent PTK inhibitor erbstatin 13 was then isolated from the fermentation broth of a *Streptomyces* and shown to possess a structure which also contains the "styryl" moiety (52). From the period 1985-1988 derivatives were prepared not only of erbstatin (53) but also of a wide variety of extremely potent PTK inhibitors based on other styryl-containing nuclei. These latter were synthesized in pioneering work by Shiraishi *et al.*, who employed diverse functionalities both on the aryl ring and at the terminal vinyl positions (54-65). Some analogs (for example compound 14 (54)) achieved IC_{50} values in the sub-micromolar range. Subsequently three broad families of derivatives based on this theme have been prepared by Levitzki *et al.*, who suggest that the name "tyrphostin" be applied to this class of compounds (66-71). Structural similarities of styryl-based PTK inhibitors to tyrosyl residues has led to the hypothesis that these agents may function as peptidomimetics which compete for tyrosyl-containing substrates at the catalytic site (71-73), a fact which is supported both by Wong's initial observations using the dehydrophenylalanine-containing peptide (22) and by the kinetics of inhibition which indicate that many compounds of this class are competitive with respect to peptide substrate (66, 68, 72, 73). Accordingly, these compounds typically exhibit a high specificity for PTKs relative to serine/threonine protein kinases (64, 74) and have also shown remarkable selectivity among different families of PTKs (66, 68-72, 75).

The activity of compounds such as 15 (69), which is a ring-constrained analog of styryl-based inhibitor 16 (68), and compound 17 (76), which is a ring-constrained mimic of 18 (68), indicate that the aryl ring and vinyl side chain of styryl-based inhibitors may be co-planar in the active conformation, similar to flavonoids. One striking feature of the flavonoid ATP inhibitors is the planarity of the benzopyrone ring system. Consistent with the ability of styryl-based inhibitors to provide partial recognition features found in flavonoids is the report that erbstatin is an effective inhibitor of some serine/threonine protein kinases where it is competitive with respect to ATP (77). The potential for a more complex mode of interaction with PTKs, other than simple mimicking of a tyrosyl-containing substrate, is also shown with piceatannol 19, which is competitive with respect to peptide substrate (41). Since piceatannol has a structural resemblance to inhibitors which are competitive with respect to peptide substrate and inhibitors which are competitive with respect to ATP (Fig. 2), it is not surprising that modifications of piceatannol can result in analogs which are competitive

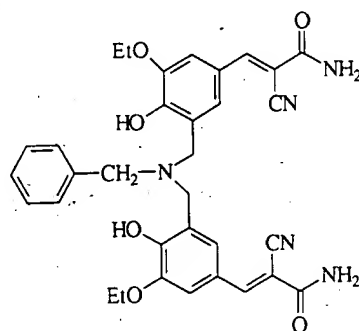


12



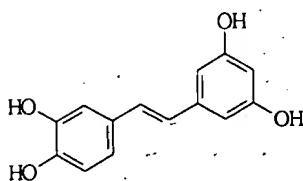
13

Erbstatin



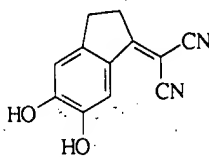
14

(type of inhibition not reported)



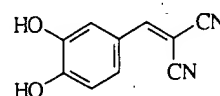
19

Piceatannol

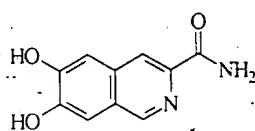


15

(type of inhibition not reported)

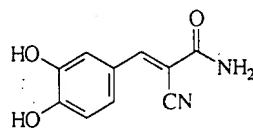


16



17

(type of inhibition not determined)



18

Fig. 3. Inhibitors which are competitive with respect to phosphoacceptor.

either with respect to ATP (compound 8) or competitive with respect to peptide substrate, depending on the modification (42, 78). The potential for multiple modes of interaction may also be indicated by unusual PTK inhibition curves obtained with certain of these inhibitors (75). Such curves could indicate sites of interaction with the enzyme which are distinct from either the ATP or peptide substrate binding sites (70). In spite of the uncertainties regarding the mechanism of action of styryl-based PTK inhibitors, these agents are proving to be useful pharmacological tools (79-86) and may provide potentially valuable antiproliferative agents (70, 71, 87-90).

Bisubstrate analogs as inhibitors at the catalytic site

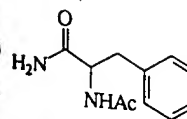
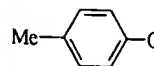
Bisubstrate analogs incorporate structural aspects of both ATP and tyrosyl-containing substrate. Theoretically by presenting a greater array of recognition features, these agents have the potential of displaying higher affinity than compounds which interact singly at either the ATP or substrate binding sites. Such compounds are based on the assumption that both ATP and tyrosyl-containing substrate are simultaneously bound to the PTK in a ternary complex (21,

22), being separated by a small distance (spannable by a triphosphate chain). Many bisubstrate analogs designed as inhibitors of PTKs emphasize the ATP moiety ("nucleoside-based"), having a fully formed nucleoside with the substrate-tyrosyl portion represented to a lesser extent by a simple aromatic ring tethered by an appropriate "polyphosphate-like" chain at some distance from the nucleoside. An alternative approach to the design of bisubstrate analogs emphasizes the substrate-tyrosyl portion ("phosphoacceptor-based"), relegating the ATP portion to a more minor constituent such as a phosphonate group.

Nucleoside-based bisubstrate inhibitors

Nucleoside-based bisubstrate analogs rely on structural elements of ATP to provide kinase affinity, with selectivity towards PTKs being provided by additional structural elements which mimic a tyrosyl moiety. Analogs have been designed which replace the highly charged triphosphate chain of ATP with functionalities more compatible with lipid transport. Nucleoside-based bisubstrate inhibitors differ principally in the nature and length of this triphosphate replace-

ment, and on one approach propionylphosphate to mimic the this analog is competitive with occurring ex interaction at it is the inte should provide this compou PTKs versus another serie which the p-phosphate ch ring separate lene chains c or ester linka bition is indee pears to occu ing at the sub above (91), th versus serine signed to exa and structural binding site, a osine via pol spite of the moieties endo pound 22), ne polyphosphat ues, indicatin

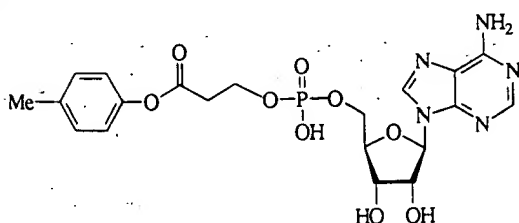


ment, and on the moiety utilized to mimic the tyrosyl unit. In one approach the triphosphate chain was replaced with a propionylphosphate and utilized a simple *p*-toluoyloxy group to mimic the tyrosyl ring (compound **20**) (91). In PTK assays this analog is competitive with respect to ATP and uncompetitive with respect to substrate, indicating that binding is occurring exclusively at the ATP site (with no corresponding interaction at the substrate binding site as intended). Since it is the interaction with the substrate binding site which should provide selectivity towards PTKs, it is surprising that this compound exhibits considerable selectivity towards PTKs *versus* a serine/threonine kinase (91) (Fig. 4). In another series of nucleoside-based bisubstrate inhibitors in which the *p*-sulfonylbenzoyl moiety is used to mimic the phosphate chain, the tyrosyl portion is provided by a phenyl ring separated from the *p*-sulfonylbenzoyl group via methylene chains of varying length and joined by either an amide or ester linkage (for example, compound **21**) (92). PTK inhibition is independent of spacer chain length and binding appears to occur at the ATP binding site with no apparent binding at the substrate-tyrosyl site. Unlike analog **20** described above (91), there appears to be no selectivity towards PTKs *versus* serine/threonine kinases. In separate studies designed to examine the role of both phosphate chain length and structural determinants for interaction with the tyrosyl binding site, a variety of substituents were tethered to adenosine via polyphosphate chains of varying length (93). In spite of the fact that some analogs employ tyrosine-like moieties endowed with significant peptide character (compound **22**), neither the nature of the tyrosyl mimetic nor the polyphosphate chain length significantly affected IC_{50} values, indicating that recognition and binding was occurring

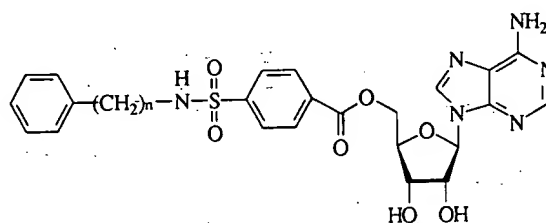
principally at the ATP binding site. To date nucleoside-based bisubstrate analogs have been unable to effectively provide simultaneous recognition of both ATP and tyrosyl substrate binding sites, and further work will be required to demonstrate the potential utility of this class of compounds.

Phosphoacceptor-based bisubstrate inhibitors

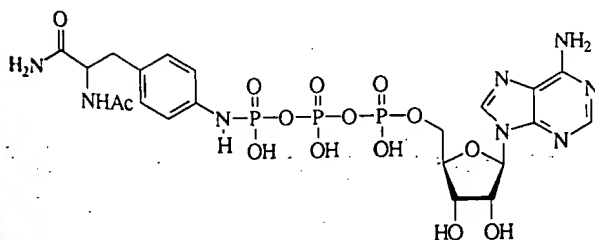
Phosphoacceptor-based, in contrast to nucleoside-based, bisubstrate inhibitors principally mimic the tyrosyl-containing substrate, with minimal structural features directed towards the ATP binding site. As has been shown above, certain styryl-based compounds, such as erbstatin, inhibit PTKs in a manner which is competitive with respect to tyrosyl-containing substrate, indicating that such compounds may have the capacity to mimic tyrosyl residues at the active site. Analogs have been prepared which incorporate phosphonate or hydroxymethylphosphonate functionality into such styryl-based molecules, thereby allowing potential interaction with both substrate and ATP binding sites. The first report of such an approach utilized a simple naphthalene ring to mimic the styryl-nucleus and included a 2-hydroxymethylphosphonate group to interact with features at the catalytic site constitutively involved in the transfer of the gamma-phosphate from ATP (compound **23**) (94). This analog showed moderate inhibition against the insulin receptor PTK and the EGFR PTK, and was somewhat selective for PTKs *versus* serine/threonine kinases. However, kinetic analysis indicated that inhibition was competitive with respect to tyrosyl-containing substrate and noncompetitive with respect to ATP, with binding being somewhat coopera-



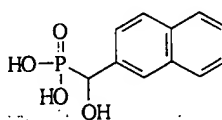
20



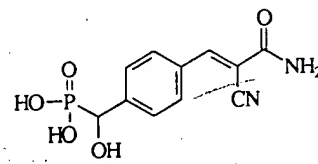
21



22



23



24

Fig. 4. Bisubstrate inhibitors.

tive with respect to ATP. One interpretation of these results is that the active site can accommodate four phosphate groups (94). Protected derivatives of **23** were additionally prepared to enhance transport across cellular membranes. While inactive in their protected form, such prodrugs can be converted to the active phosphonate within cells and have been shown to be antiproliferative in several tumor cell lines (94). In a separate study based on a similar rationale, the hydroxymethylphosphonate group was incorporated into a variety of more fully elaborated styryl-based compounds (for example, compound **24**) (95). While it would be expected that such compounds should possess greater inhibitory activity than the simple naphthalene-based compound **23**, surprisingly all analogs proved to be inactive. Since these latter agents more closely represent the structural features which the naphthalene-based compound seeks to mimic, their inactivity may indicate either that the naphthalene-based analog **23** is not binding in a manner analogous to the styryl-based inhibitors, or that the styryl-based class of inhibitors, including erbstatin, do not interact at the catalytic site in a manner which is analogous to tyrosyl residues (95). Additional work is ongoing to more fully examine these questions. (See Addendum)

Inhibitors of substrate recognition outside the catalytic site

Low affinities and general lack of a well defined correlation between amino acid sequences of small tyrosine-containing peptides and affinity towards PTKs is suggestive that higher order protein interactions outside the catalytic site are constitutively involved in substrate binding. As previously mentioned, semi-conserved noncatalytic domains termed "src homology regions" 2 and 3 (SH2 and SH3) are found in a number of PTKs and in cellular proteins and substrates which associate with PTKs. These regions regulate PTK-substrate interaction by recognizing and binding to specific peptide sequences containing phosphorylated tyrosines (15). Such phosphorylated tyrosines may reside within the PTK and result from autophosphorylation or phosphorylation by other kinases, or may be located within the substrate. A phosphotyrosine-containing consensus sequence derived from PTK autophosphorylation sites has been proposed for recognition and binding to an SH2 domain of one important PTK substrate, phosphatidylinositol-3 kinase (PI3 kinase) (96). Conceptually, agents which interfere with this association have the potential of inhibiting PTK function by preventing the recognition and binding of substrate. A phosphotyrosine-containing 20-amino acid sequence encompassing an autophosphorylation site of the PDGF PTK has been shown to selectively block the association of PI2 kinase with the PDGF kinase (97). More recently this recognition sequence has been narrowed to a 5 amino acid phosphotyrosine-containing peptide which has the capacity to selectively block the binding of the PI3 kinase (98). The high structural specificity for this inhibition (homologous peptides possessing single amino acid alterations were inactive) (98) and the fact that SH2 domains of different substrates exhibit differential recognition of PTKs (15) could endow SH2-based inhibitors with a very high degree of selectivity.

While the above approach is based on phosphotyrosine-containing sequences which are recognized by SH2 domains, an alternate approach involves the preparation of analogs which are modeled on portions of the SH2 domain itself. In this regard a 21 amino acid sequence derived from a segment of the SH2 region of the v-src PTK was found to inhibit a variety of PTKs in a manner which was not competitive with respect to either substrate or ATP. In addition, it showed no activity against serine/threonine kinases (99). While the mechanism of this inhibition is not understood, it demonstrates the potential utility of designing SH2-based inhibitors which mimic portions of the SH2 domain itself and which do not contain phosphotyrosine residues.

Agents which inhibit by other mechanisms

A variety of agents have been shown to inhibit PTKs by mechanisms of action which have not been demonstrated or which do not fall explicitly within categories covered above. A brief description of some of these compounds will be provided below (Fig. 5).

Herbimycin A

Herbimycin A **25**, a benzoquinoid ansamycin antibiotic isolated from a culture of a *Streptomyces* has been shown to reduce the phosphotyrosine content, effectively reverse the transformed phenotype of cells bearing a number of oncogenic PTKs (100, 101), and reestablish contact inhibition (102). While this effect has been shown to be reversible in living cells (102), irreversible inhibition of immunopurified PTK preparations has been observed, although it can be abrogated by the presence of sulfhydryl reagents (103). Such evidence may indicate covalent interaction of herbimycin A with sulfhydryl groups on the PTK. Additional studies have shown that PTKs can be protected from herbimycin A induced inactivation by pretreatment with other sulfhydryl-reactive reagents which do not themselves inactivate the kinase. This latter evidence suggests that herbimycin A may bind to sulfhydryl groups which are situated near the catalytic site, but which are not required for kinase activity (104). While only partially cytotoxic at concentrations required to reverse transformation, herbimycin A has potential clinical utility in its ability to establish synchronous growth following its removal. Such synchronism when used in combination therapy could potentially enhance the effectiveness of other chemotherapeutic agents directed against DNA synthesis (105).

Thiazolidine-diones

Several derivatives of thiazolidine-diones (for example, compound **26**) have been shown to specifically inhibit PTKs (EGFR and c-src) at concentrations where serine/threonine kinases are not inhibited, and to have some selectivity among different PTKs (v-abl is not inhibited as effectively) (106). These agents are able to inhibit PTKs *in vitro* in intact cells and to inhibit growth factor-induced mitogenesis. Inhibition is not competitive with respect to ATP, indicating that

the com-
zolidine-
PTK-sel-

Epidersta

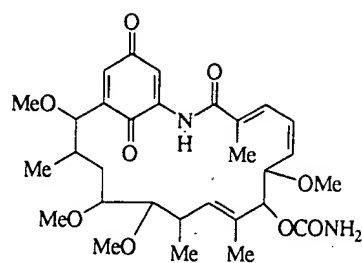
Epider-
mide anti-
myces an-
epiderma-
idine-dio-
tivity in vi-
is unknow

(+)-Aeropl

The natu-
nin-1 **28** is
in receptor
eration of H
has also b
against tum
tostatic ag
mechanism
(+)-aeropl
lead in the c

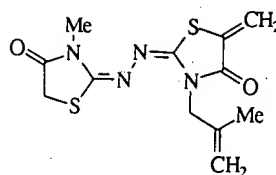
Phenazocin

It is know
plastic prop
pend on th
sine-contain

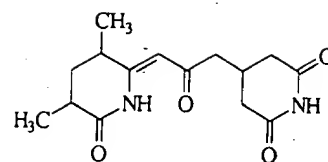


25

Herbimycin A

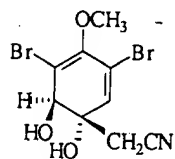


26



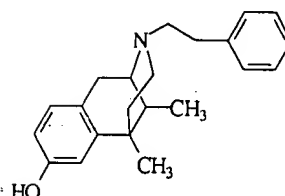
27

Epiderstatin



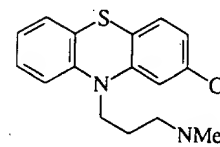
28

(+)-Aeropylsinin-1



29

(+/-)-Phenazocine



30

Chlorpromazine

Fig. 5. Miscellaneous inhibitors.

the compounds were not functioning as ATP analogs. Thiazolidine-diones represent an interesting new class of PTK-selective inhibitors.

Epiderstatin

Epiderstatin **27**, a piperidinedione member of the glutarimide antibiotics (107), was isolated from a strain of *Streptomyces* and shown to inhibit mitogenic activity induced by epidermal growth factor (108). Unlike the thiazolidine-diones, epiderstatin does not inhibit EGFR kinase activity *in vitro* (108) and its mechanism of action in living cells is unknown.

(+)-Aeropylsinin-1

The naturally occurring tyrosine metabolite (+)-aeropylsinin-1 **28** is a highly potent inhibitor of EGFR kinase activity in receptor preparations and blocks EGF-dependent proliferation of human breast cancer cells (109). The compound has also been shown to possess potent cytotoxic activity against tumor cell lines at concentrations which are only cytostatic against non-transformed cells (110). While the mechanism of EGFR inhibition has not been explored, (+)-aeropylsinin-1 may potentially represent an interesting lead in the development of PTK inhibitors.

Phenazocine

It is known that certain opioid compounds exhibit antineoplastic properties. Since the analgesic action of opiates depends on their ability to mimic small endogenous tyrosine-containing peptides, a study was undertaken to

examine whether the observed antineoplastic effects of opiates was associated with inhibition of PTKs (111). Of 13 opiates examined for inhibition of EGFR kinase activity, only (+)-phenazocine **29** was found to be active, possessing a potency approaching that of epiderstatin in the assay. The mechanism of inhibition was not examined.

Halomethyl ketones

A variety of halomethyl ketone derivatives of amino acids and peptides have been shown to inhibit PTKs. One particularly well studied derivative, Boc-Leu-CH₂-Br, inhibits the EGFR PTK in a manner which does not apparently affect either the binding of EGF or ATP to the receptor. Interestingly, binding of EGF significantly protected against the effects of the inhibitor. Inhibition was selective for PTK *versus* serine/threonine kinases and showed some discrimination between three different PTKs (112).

Lipid-active inhibitors

Most PTKs are intimately associated with lipid environments. Catalytic activity can be modulated by agents which affect this interaction, a phenomenon which is shared by other lipid-bound kinases such as protein kinase C (113). Chlorpromazine **30** is an effective inhibitor of the src PTK, and evidence suggests that it exerts this inhibitory activity through interaction with phospholipids (114). Such inhibition is nonspecific and extends to other kinases such as protein kinase C (113). Sphingosines are inhibitory to both PTKs and protein kinase C; however, *N*-substituted sphingosines are stimulatory to c-src and v-src (115), indicating a more complex interaction than simple membrane disruption. Retinoic acid has also been shown to inhibit EGF-stimulated cell

proliferation in a manner which parallels its inhibition of EGFR kinase activity. The inhibition of retinoic acid on EGFR does not appear to be mediated through modulation of protein kinase C and may arise from alterations in EGFR structure (116). While the potential of lipid-active agents such as TPK inhibitor-based therapeutics may be limited by lack of specificity, patent protection has been applied for a series of polyunsaturated long chain fatty acid-containing amino acid derivatives as antineoplastic agents which are claimed to function by inhibiting PTKs (117).

Conclusions

Agents which inhibit the function of PTKs have potential both as antiproliferative therapeutics and as probes for elucidating the role of these enzymes in signal transduction. The lack of three-dimensional structural information regarding PTKs, and the ability of inhibitors which are closely related in structure to interact with PTKs by different modes, complicates the rational design of new PTK inhibitors. In spite of these limitations, significant advances in the area of analog design are predictable. These, in turn, should provide the basis for arriving at a greater understanding of the functional relationships of PTKs themselves.

Addendum

Two significant developments worthy of note appeared in print following the initial submission of this review. The preparation of sulfonylbenzoyl-nitrostyrenes, which were designed as "phosphoacceptor-based bisubstrate" PTK inhibitors (using the terminology of this review) were reported (118). The approach, which was conceptually similar to that employed in the design of compound 24 (95), utilized the sulfonylbenzoyl moiety to mimic the phosphate chain of ATP, in combination with a nitrostyryl group, which mimicked the tyrosyl portion of the substrate. Some of the resulting analogs were extremely potent, with one compound exhibiting an IC_{50} against EGFR PTK of 54 nM. This report is important in that it highlights the utility of phosphoacceptor-based bisubstrate compounds as rationally designed PTK inhibitors. The discrepancy between the very good activity of the sulfonylbenzoyl-nitrostyryl as compared to the inactivity of the phosphonomethyl-styryls (such as 24) also emphasizes the importance of the kind of functionality used to mimic the ATP phosphate chain.

A second important development originates from a detailed kinetic analysis of the interaction of lavendustin A 10 and a closely related analog with the EGFR PTK (119). While initial reports indicated that lavendustin A was competitive with respect to ATP and non-competitive with respect to peptide substrate (44), this more detailed kinetic analysis indicated that lavendustin A is a "mixed type inhibitor", affecting the binding of both ATP and tyrosyl-substrate. This is significant in that it suggests the presence of a binding site for lavendustin A which is distinct from the binding site of either ATP or tyrosyl-substrate. The existence of such an "auxiliary binding site(s)" could explain unusual inhibition curves found with other PTK inhibitors (70, 75) and has im-

portant implications in the rational design of mechanism-based PTK inhibitors.

Acknowledgements

Appreciation is expressed to Drs. Victor Marquez and Peter Roller for critical reading of this manuscript.

References

- Hunter, T. *Protein modification: Phosphorylation on tyrosine residues*. Curr Opin Cell Biol 1989, 1: 1168-81.
- White, M.F. *Structure and function of tyrosine kinase receptors*. J Bioenerg Biomembrane 1991, 23: 63-82.
- Ullrich, A., Schlessinger, J. *Signal transduction by receptors with tyrosine kinase activity*. Cell 1990, 61: 203-12.
- Veillette, A., Bolen, J.B. *Src-related protein tyrosine kinases*. In: Oncongenes. Benz, C., Liu, E. (Eds.). Kluwer Academic Publishers: Boston 1989, 121-42.
- Di Marco, E., Pierce, J.H., Aaronson, S.A., Di Fiore, P.P. *Mechanisms by which EGF receptor and TGF- α contribute to malignant transformation*. Nat Immun Cell Growth Regul 1990, 9: 209-21.
- Gullick, W.J. *Prevalence of aberrant expression of the epidermal growth factor receptor in human cancers*. Brit Med Bull 1991, 47: 87-98.
- Tritton, T.R., Hickman, J.A. *How to kill cancer cells: Membranes and cell signaling as targets in cancer chemotherapy*. Cancer Cells 1990, 2: 95-105.
- Comoglio, P.M., Di Renzo, M.F., Gaudino, G., Ponzetto, C., Prat, M. *Tyrosine kinase and control of cell proliferation*. Amer Rev Resp Dis 1990, 142: S16-19.
- Powis, G. *Signalling targets for anticancer drug development*. Trends Pharmacol Sci 1991, 12: 188-94.
- Parsons, J.T., Weber, M.J. *Genetics of src: Structure and functional organization of a protein tyrosine kinase*. Curr Top Microbiol Immunol 1989, 147: 79-127.
- Schlessinger, J. *Signal transduction by allosteric receptor oligomerization*. Trends Pharmacol Sci 1988, 13: 443-7.
- Margolis, B., Li, N., Koch, A., Mohammadi, M., Hurwitz, D.R., Zilberstein, A., Ullrich, A., Pawson, T., Schlessinger, J. *The tyrosine phosphorylated carboxyterminus of the EGF receptor is a binding site for GAP and PLC- γ* . EMBO J 1990, 9: 4375-80.
- Hsu, C.-Y., Hurwitz, D.R., Mervic, M., Zilberstein, A. *Autophosphorylation of the intracellular domain of the epidermal growth factor receptor results in different effects on its tyrosine kinase activity with various peptide substrates - Phosphorylation of peptides representing tyr(P) sites of phospholipase C- γ* . J Biol Chem 1991, 266: 603-8.
- Varmus, H., Hirai, H., Morgan, D., Kaplan, J., Bishop, J.M. *Function, location, and regulation of the src protein-tyrosine kinase*. In: Genetic Basis for Carcinogenesis: Tumor Suppressor Genes and Oncogenes. Knudson, A.G. (Ed.). Japan Sci Soc Press: Tokyo 1990, 63-70.
- Koch, C.A., Anderson, D., Moran, M.F., Ellis, C., Pawson, T. *SH2 and SH3 domains: Elements that control interactions of cytoplasmic signaling proteins*. Science 1991, 252: 668-74.
- O'Brien, M.C., Fukui, Y., Hanafusa, H. *Activation of proto-oncogene p60^{c-src} by point mutations in the SH2 domain*. Mol Cell Biol 1990, 10: 2855-62.
- Potts, W.M., Reynolds, A.B., Lansing, T.J., Parsons, T. *Activation of pp60^{c-src} transforming potential by mutations altering the*

structure of
Oncogene

18. Kenyon
Rev 1987, 7

19. Larson,
py. In: Annu
(Ed.). Acad

20. Johnson
anticancer a
25. Bristol, J
136.

21. Erneux,
phosphoryla
nase of A-43

22. Wong, T.
tensin phosph
sarcoma viru

23. Knighton,
N.-H., Taylor,
subunit of cyc
nase. Scienc

24. Hanks, S.
Conserved fe
mains. Scienc

25. Kamps, M.
cogenic tyrosi
have homolog

26. Barnekow,
ing activities
pp60(v-src) ar
Rep 1983, 3: 1

27. Cody, V., I
Plant Flavono
and Medicinal

28. Hagiwara, I.
H. Differential
kinases and se
1988, 37: 2987

29. Geahlen, R.
Inhibition of pr
lated compound

30. Akiyama, T.
S., Itoh, N., Shi
tyrosine-specifi

31. Ogawara, H.
Seoda, Y. Inhibi
isoflavones and

32. Cushman, M.
thesis and prote
analogues. J Me

33. Naik, R.G., L
Czech, J. Prepa
tives as antitum
61928c.

34. Shida, Y., Sa
nolflavone deriva
374,789 (June 2

35. Linossier, C.
of action in NIH-3
tyrosine kinase a

36. Davis, R.J., C
receptor tyrosine

- structure of an amino terminal domain containing residues 90-95. *Oncogene Res* 1988, 3: 343-55.
18. Kenyon, G.L., Garcia, G.A. *Design of kinase inhibitors*. *Med Res Rev* 1987, 7: 389-416.
19. Larson, E.R., Fischer, P.H. *New approaches to antitumor therapy*. In: *Annual Reports in Medicinal Chemistry*, Vol. 24. Allen, R.C. (Ed.). Academic Press, Inc., San Diego, CA: 1989, 125-6.
20. Johnson, R.J., Hertzberg, R.P. *Mechanism-based discovery of anticancer agents*. In: *Annual Reports in Medicinal Chemistry*, Vol. 25. Bristol, J.A. (Ed.). Academic Press, Inc., San Diego, CA: 1990, 136.
21. Erneux, C., Cohen, S., Garbers, D.L. *The kinetics of tyrosine phosphorylation by the purified epidermal growth factor receptor kinase of A-431 cells*. *J Biol Chem* 1983, 258: 4137-42.
22. Wong, T.W., Goldberg, A.R. *Kinetics and mechanism of angiotensin phosphorylation by the transforming gene product of Rous sarcoma virus*. *J Biol Chem* 1984, 259: 3127-31.
23. Knighton, D.R., Zheng, J., Ten Eyck, L.F., Ashford, V.A., Xuong, N.-H., Taylor, S.S., Sowadski, J.M. *Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase*. *Science* 1991, 253: 407-14.
24. Hanks, S.K., Quinn, A.M., Hunter, T. *The protein kinase family: Conserved features and deduced phylogeny of the catalytic domains*. *Science* 1988, 241: 42-52.
25. Kamps, M.P., Taylor, S.S., Sefton, B.M. *Direct evidence that oncogenic tyrosine kinases and cyclic AMP-dependent protein kinase have homologous ATP-binding sites*. *Nature* 1984, 310: 589-92.
26. Barnekow, A. *Effect of several nucleotides on the phosphorylating activities of the Rous-sarcoma-virus transforming protein pp60(v-src) and its cellular protein homologue, pp60(c-src)*. *Biosci Rep* 1983, 3: 153-62.
27. Cody, V., Middleton, E. Jr., Harborne, J.B., Beretz, A. (Eds.). *Plant Flavonoids in Biology and Medicine II*. Biochemical, Cellular, and Medicinal Properties. Alan R. Liss, Inc.: New York 1987.
28. Hagiwara, M., Inoue, S., Tanaka, T., Nunoki, K., Ito, M., Hidaka, H. *Differential effects of flavonoids as inhibitors of tyrosine protein kinases and serine/threonine protein kinases*. *Biochem Pharmacol* 1988, 37: 2987-92.
29. Geahlen, R.L., Koonchanok, N.M., McLaughlin, J.L., Pratt, D.E. *Inhibition of protein-tyrosine kinase activity by flavonoids and related compounds*. *J Nat Prod* 1989, 52: 982-6.
30. Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M., Fukami, Y. *Genistein, specific inhibitor of tyrosine-specific protein kinases*. *J Biol Chem* 1987, 262: 5592-5.
31. Ogawara, H., Akiyama, T., Watanabe, S., Itoh, N., Kabori, M., Seoda, Y. *Inhibition of tyrosine protein kinase activity by synthetic isoflavones and flavones*. *J Antibiot* 1989, 42: 340-3.
32. Cushman, M., Nagarathnam, D., Burg, D.L., Geahlen, R.L. *Synthesis and protein-tyrosine kinase inhibitory activities of flavonoid analogues*. *J Med Chem* 1991, 34: 798-806.
33. Naik, R.G., Lal, B., Rupp, R.H., Sedlacek, H.H., Dickneite, G., Czech, J. *Preparation and use of 4H-1-benzopyran-4-one derivatives as antitumor agents*. *DE 3,836,676* (May 3, 1990), CA 114: 61928c.
34. Shida, Y., Sagaya, T., Gomi, K., Kasai, M., Morimoto, M. *5-Aminoflavone derivatives and their preparation as antitumor agents*. *EP 374,789* (June 27, 1990).
35. Linossier, C., Pierre, M., Le Pecq, J.B., Pierre, J. *Mechanisms of action in NIH-3T3 cells of genistein, an inhibitor of EGF receptor tyrosine kinase activity*. *Biochem Pharmacol* 1990, 39: 187-93.
36. Davis, R.J., Czech, M.P. *Amiloride directly inhibits growth factor receptor tyrosine kinase activity*. *J Biol Chem* 1985, 260: 2543-51.
37. Sparks, R.L., Pool, T.B., Smith, N.K.R., Cameron, I.L. *Effects of amiloride on tumor growth and intracellular element content of tumor cells in vivo*. *Cancer Res* 1983, 43: 73-7.
38. Ruegg, U.T., Burgess, G.M. *Staurosporine, K-252 and UNC-01: Potent but nonspecific inhibitors of protein kinases*. *Trends Pharmacol Sci* 1989, 10: 218-20.
39. Takahashi, H., Osada, H., Uramoto, M., Isono, K. *A new inhibitor of protein kinase-C, RK-286C (4'-demethylamino-4'-hydroxystaurosporine) 2. Isolation, physico-chemical properties and structure*. *J Antibiot* 1990, 43: 168-73.
40. Herbert, J.M., Seban, E., Maffrand, J.P. *Characterization of specific binding sites for [H-3]-staurosporine on various protein kinases*. *Biochem Biophys Res Commun* 1990, 171: 189-95.
41. Geahlen, R.L., McLaughlin, J.L. *Piceatannol (3,4,3',5'-Tetrahydroxy-trans-stilbene) is a naturally occurring protein-tyrosine kinase inhibitor*. *Biochem Biophys Res Commun* 1989, 165: 241-5.
42. Cushman, M., Nagarathnam, D., Gopal, D., Geahlen, R.L. *Synthesis and evaluation of new protein-tyrosine kinase inhibitors. Part 1. Pyridine-containing stilbenes and amides*. *Bioorg Med Chem Lett* 1991, 1: 211-14.
43. Donella-Deana, A., Monti, E., Pinna, L.A. *Inhibition of tyrosine protein kinases by the antineoplastic agent adriamycin*. *Biochem Biophys Res Commun* 1989, 160: 1309-15.
44. Onoda, T., Iinuma, H., Sasaki, Y., Hamada, M., Isshiki, K., Nagawara, H., Takeuchi, T. *Isolation of a novel tyrosine kinase inhibitor, lavendustin A, from Streptomyces griseolavendus*. *J Nat Prod* 1989, 52: 1252-7.
45. Geahlen, R.L., Harrison, M.L. *Protein-tyrosine kinases*. In: *Peptides and Protein Phosphorylation*. Kemp, B.E. (Ed.). CRC Press, Inc.: Boca Raton, FL 1990, 239-53.
46. Braun, S., Raymond, W.E., Racker, E. *Synthetic tyrosine polymers as substrates and inhibitors of tyrosine-specific protein kinases*. *J Biol Chem* 1984, 259: 2051-4.
47. Shoelson, S.E., White, M.F., Kahn, C.R. *Nonphosphorylatable substrate analogs selectively block autophosphorylation and activation of the insulin receptor, epidermal growth factor receptor and pp60v-src kinases*. *J Biol Chem* 1989, 264: 7831-6.
48. Cushman, M., Chinnasamy, P., Chakraborti, A.K., Jurayj, J., Geahlen, R.L., Haugwitz, R.D. *Synthesis of [beta-(4-pyridyl-1-oxide)-L-alanine]-angiotensin I as a potential suicide substrate for protein-tyrosine kinases*. *Int J Pept Protein Res* 1990, 36: 538-43.
49. Yuan, C.J., Jakes, S., Elliot, S., Graves, D.J. *A rationale for the design of an inhibitor of tyrosyl kinase*. *J Biol Chem* 1990, 265: 16205-9.
50. Shechter, Y., Yaish, P., Chorev, M., Gilon, C., Braun, S., Levitzki, A. *Inhibition of insulin-dependent lipogenesis and anti-lipolysis by protein tyrosine kinase inhibitors*. *EMBO J* 1989, 8: 1671-6.
51. Goto, Y., Maeda, H., Fujeda, S. *Tyrosine kinase inhibitors and anticancer agents containing 3-(hydroxyphenyl)pyruvic and lactic acid derivatives*. *JP 02,142,725* (May 31, 1990), CA 113: 152052h.
52. Nakamura, H., Iitaka, Y., Imoto, M., Isshiki, K., Naganawa, H., Takeuchi, T., Umezawa, H. *The structure of an epidermal growth factor-receptor kinase inhibitor, erbstatin*. *J Antibiot* 1986, 39: 314-15.
53. Isshiki, K., Imoto, M., Sawa, T., Umezawa, K., Takeuchi, T., Umezawa, H., Tsuchida, T., Yoshioka, T., Tatsuta, K. *Inhibition of tyrosine protein kinase by synthetic erbstatin analogs*. *J Antibiot* 1987, 40: 1209-10.
54. Shiraishi, T., Kameyama, K., Domoto, T., Shimada, Y., Hidaka, T., Katsumi, I., Watanabe, K. *Preparation of tribenzylamine derivatives as tyrosine kinase inhibitors*. *JP 63,141,955* (June 14, 1988), CA 109: 210674t.
55. Shiraishi, T., Shimada, Y., Imai, N., Katsumi, I., Watanabe, K. *3,5-Diisopropyl-4-hydroxystyrene derivatives as tyrosine kinase inhibitors in cancer treatment*. *JP 39,522* (Feb. 20, 1987), CA 106: 201757h.

56. Shiraishi, T., Shimada, Y., Katsumi, I., Yamashita, K., Watanabe, K. 3,5-Di-tertiary-butyl-4-hydroxystyrene derivatives as anti-tumor agents. JP 62 39,523 (Feb. 20, 1987), CA 106: 201758j.
57. Shiraishi, T., Domoto, T., Imai, N., Katsumi, I., Watanabe, K. Styrene derivatives a tyrosine kinase inhibitors in cancer therapy. JP 62 42,923 (Feb. 24, 1987), CA 106: 201762f.
58. Shiraishi, T., Domoto, T., Imai, N., Katsumi, I., Yamashita, K. 4-Alkoxyterene derivatives as tyrosine kinase inhibitors in cancer therapy. JP 62 42,925 (Feb. 24, 1987), CA 106: 201763g.
59. Shiraishi, T., Shimada, Y., Fuse, Y., Imai, N., Yamashita, K., Yamashita, T. L-Benzylidene-gamma-butyrolactone or gamma-butyrolactam derivatives. JP 62 39,564 (Feb. 20, 1987), CA 107: 96584v.
60. Shiraishi, T., Domoto, T., Shimada, Y., Imai, N., Kondo, H., Katsumi, I., Hidaka, T., Watanabe, K. Preparation of alpha-cyanoacrylamide derivatives as antibacterials and tyrosine kinase inhibitors. JP 62 39,558 (Feb. 20, 1987), CA 107: 58668t.
61. Imai, N., Shiraishi, T., Katsumi, I., Yamashita, K., Hosoe, K., Arikawa, Y., Watanabe, K. 5-(3,5-Diisopropyl-4-hydroxybenzylidene)-2-amino-4-oxothiazole derivatives useful as allergy inhibitors. JP 62 29,579 (Feb 7, 1987), CA 106: 213937e.
62. Imai, N., Shiraishi, T., Katsumi, I., Yamashita, K., Arikawa, Y., Yamashita, T. Diisopropylbenzylidene-substituted heterocycles. JP 62 29,570 (Feb 7, 1987), CA 106: 213918z.
63. Kameyama, K., Shiraishi, T., Domoto, T., Katsumi, I., Hidaka, T. Preparation of alpha-cyanocinnamamide derivatives. JP 63,222,153 (Sept. 16, 1988), CA 110: 212392u.
64. Shiraishi, T., Kameyama, K., Domoto, T., Imai, N., Shimada, Y., Arikawa, Y., Hosoe, K., Kawatsu, M., Katsumi, I. Preparation of hydroxystyrene derivatives as pharmaceuticals. WO 88 07,035 (Sept. 22, 1988), CA 110: 57296v.
65. Shiraishi, T., Domoto, T., Imai, N., Shimada, Y., Watanabe, K. Specific inhibitors of tyrosine-specific protein kinase, synthetic 4-hydroxycinnamamide derivatives. Biochem Biophys Res Commun 1987, 147: 322-8.
66. Yaish, P., Gazit, A., Gilon, C., Levitzki, A. Blocking of EGF-dependent cell proliferation by EGF receptor kinase inhibitors. Science 1988, 242: 933-5.
67. Levitzki, A., Gilon, C., Chorev, M., Gazit, A. Benzylidene and cinnamylidene-malonitrile derivatives for the inhibition of proliferative processes in mammalian cells. EP 0 322 738 A2 (Feb. 5, 1989), CA 112: 172316e.
68. Gazit, A., Yaish, P., Gilon, C., Levitzki, A. Tyrphostins I: Synthesis and biological activity of protein tyrosine kinase inhibitors. J Med Chem 1989, 32: 2344-52.
69. Gazit, A., Osherov, N., Posner, I., Yaish, P., Poradosu, E., Gilon, C., Levitzki, A. Tyrphostins. 2. Heterocyclic and alpha-substituted benzylidenemalonitrile tyrphostins as potent inhibitors of EGF receptor and ErbB2/neu tyrosine kinases. J Med Chem 1991, 34: 1896-907.
70. Levitzki, A., Gilon, C. Tyrphostins as molecular tools and potential antiproliferative drugs. Trends Pharmacol Sci 1991, 12: 171-4.
71. Levitzki, A. Tyrphostins-Potential antiproliferative agents and novel molecular tools. Biochem Pharmacol 1990, 40: 913-18.
72. Shiraishi, T., Owada, M.K., Tatsuka, M., Yamashita, T., Watanabe, K., Kakunaga, T. Specific inhibitors of tyrosine-specific protein kinases: Properties of 4-hydroxycinnamamide derivatives in vitro. Cancer Res 1989, 49: 2374-8.
73. Imoto, M., Umezawa, K., Isshiki, K., Kunimoto, S., Sawa, T., Takeuchi, T., Umezawa, H. Kinetic studies of tyrosine kinase inhibition by erbstatin. J Antibiot 1987, 40: 1471-3.
74. Shiraishi, T., Kameyama, K., Imai, N., Domoto, T., Katsumi, I., Watanabe, K. Specific inhibitors of tyrosine-specific protein kinase. I. Synthesis and inhibitory activities of alpha-cyanocinnamamides. Chem Pharm Bull 1988, 36: 974-81.
75. Burke, T.R. Jr., Li, Z.-H., Bolen, J.B., Marquez, V.E. Structural influences of styryl-based inhibitors on epidermal growth factor receptor and p56^{lck} tyrosine-specific protein kinases. Bioorg Med Chem Lett 1991, 1: 165-8.
76. Burke, T.R. Jr., Lim, B., Escobedo, J., Williams, L.T., Marquez, V.E. Carboxynaphthyl, quinolyl and -isoquinolyl compounds as bicyclic ring-constrained analogues of styryl-based protein tyrosine kinase inhibitors. Fourth Chemical Congress of North America: New York 1991, MEDI 24.
77. Bishop, W.R., Petrin, J., Wang, L., Ramesh, U., Doll, R.J. Inhibition of protein kinase C by the tyrosine kinase inhibitor erbstatin. Biochem Pharmacol 1990, 40: 2129-35.
78. Cushman, M., Nagarathnam, D., Gopalan, V., Geahlen, R.L. Synthesis and evaluation of new protein-tyrosine kinase inhibitors. Part 2. Phenylhydrazones. Bioorg Med Chem Lett 1991, 1: 215-18.
79. Umezawa, K., Hori, T., Tajima, H., Imoto, M., Isshiki, K., Takeuchi, T. Inhibition of epidermal growth factor-induced DNA synthesis by tyrosine kinase inhibitors. FEBS Lett 1990, 260: 198-200.
80. Shiraishi, T., Owada, M.K., Tatsuka, M., Fuse, Y., Watanabe, K., Kakunaga, T. A tyrosine-specific protein kinase inhibitor, alpha-cyano-3-ethoxy-4-hydroxy-5-phenylthiomethylcinnamamide, blocks the phosphorylation of tyrosine kinase substrate in intact cells. Jpn J Cancer Res 1990, 81: 645-52.
81. Posner, I., Gazit, A., Gilon, C., Levitzki, A. Tyrphostins inhibit the epidermal growth factor receptor-mediated breakdown of phosphoinositides. FEBS Lett 1989, 257: 287-91.
82. Salari, H., Duronio, V., Howard, S.L., Demos, M., Jones, K., Reany, A., Hudson, A.T., Pelech, S.L. Erbstatin blocks platelet activating factor-induced protein-tyrosine phosphorylation, polyphosphoinositide hydrolysis, protein kinase-C activation, serotonin secretion and aggregation of rabbit platelets. FEBS Lett 1990, 263: 104-8.
83. Imoto, M., Shimura, N., Ui, H., Umezawa, K. Inhibition of EGF-induced phospholipase-C activation in A431 cells by erbstatin, a tyrosine kinase inhibitor. Biochem Biophys Res Commun 1990, 173: 208-11.
84. Imoto, M., Umezawa, K., Sawa, T., Takeuchi, T., Umezawa, H. In situ inhibition of tyrosine kinase by erbstatin. Biochem Int 1987, 15: 989-95.
85. Bilder, G.E., Krawiec, J.A., Mcvety, K., Gazit, A., Gilon, C., Lyall, R., Zilberstein, A., Levitzki, A., Perrone, M.H., Schreiber, A.B. Tyrphostins inhibit PDGF-induced DNA synthesis and associated early events in smooth muscle cells. Amer J Physiol 1991, 260: C721-30.
86. Faaland, C.A., Mermelstein, F.H., Hayashi, J., Laskin, J.D. Rapid uptake of tyrphostin into A431 human epidermoid cells is followed by delayed inhibition of epidermal growth factor (EGF)-stimulated EGF receptor tyrosine kinase activity. Mol Cell Biol 1991, 11: 2697-703.
87. Imoto, M., Umezawa, K., Komuro, K., Sawa, T., Takeuchi, T., Umezawa, H. Antitumor activity of erbstatin, a tyrosine protein kinase inhibitor. Jpn J Cancer Res 1987, 78: 329-32.
88. Wada, T., Toi, M., Nakamura, T., Yamamoto, A., Yanagawa, E., Toge, T., Niimoto, M., Hattori, T. Effects of erbstatin, a tyrosine kinase inhibitor, on the growth of human breast cancer cell line MCF-7. Igaku no Ayumi 1989, 150: 555-6.
89. Lyall, R.M., Zilberstein, A., Gazit, A., Gilon, C., Levitzki, A., Schlessinger, J. Tyrphostins inhibit epidermal growth factor (EGF)-receptor tyrosine kinase activity in living cells and EGF-stimulated cell proliferation. J Biol Chem 1989, 264: 14503-9.
90. Toi, M., Mukaida, H., Wada, T., Hirabayashi, N., Toge, T., Hori, T., Umezawa, K. Antineoplastic effect of erbstatin on human mammary and esophageal tumors in athymic nude mice. Eur J Cancer 1990, 26: 722-4.
91. Baginski, I., Commercon, A., Tocque, B., Colson, G., Zerial, A. Selective inhibition of tyrosine protein kinase by a synthetic multi-

substrate and
1324-30.

92. Kruse, C.
D.J., Greig,
strate inhibi
kinase. 1. J.

93. Kruse, C.
J.A., Riemar
G. Synthesis
gene-encod
1988, 31: 17

94. Saperste
Greenlee, W.
sign of a sel
effect on glu
try 1989, 28:

95. Burke, T.
nate-contain
Med Chem 1

96. Cantley,
ziani, A., Kay
tion. Cell 19

97. Escobedo
Williams, L.
let-derived g
quence com
1125-32.

98. Turck, C.
PDGF beta-
P13-kinase
sium of The
T137.

99. Sato, K.,
mi, Y. A synt
p60v-src inh
phys Res Co

100. Uehara,
change from
samycins ac
fected with F

101. Uehara,
transforming
Virology 198

102. Suzuki,
keuchi, T. I
v-Src-expres

103. Uehara,
ible inhibition
abrogation b
mun 1989, 1

104. Fukazawa,
and various
chem Biophys

105. Hori, M.
gene function

- substrate analog. *Biochem Biophys Res Commun* 1989, 165: 1324-30.
92. Kruse, C.H., Holden, K.G., Pritchard, M.L., Feild, J.A., Rieman, D.J., Greig, R.G., Poste, G. *Synthesis and evaluation of multisubstrate inhibitors of an oncogene-encoded tyrosine-specific protein kinase*. 1. *J Med Chem* 1988, 31: 1762-7.
93. Kruse, C.H., Holden, K.G., Offen, P.H., Pritchard, M.L., Feild, J.A., Rieman, D.J., Bender, P.E., Ferguson, B., Greig, R.G., Poste, G. *Synthesis and evaluation of multisubstrate inhibitors of an oncogene-encoded tyrosine-specific protein kinase*. 2. *J Med Chem* 1988, 31: 1768-72.
94. Saperstein, R., Vicario, P.P., Strout, H.V., Brady, E., Slater, E.E., Greenlee, W.J., Ondeyka, D.L., Patchett, A.A., Hangauer, D.G. *Design of a selective insulin receptor tyrosine kinase inhibitor and its effect on glucose uptake and metabolism in intact cells*. *Biochemistry* 1989, 28: 5694-701.
95. Burke, T.R. Jr., Li, Z.-H., Bolen, J.B., Marquez, V.E. *Phosphate-containing inhibitors of tyrosine-specific protein kinases*. *J Med Chem* 1991, 34: 1577-81.
96. Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., Soltoff, S. *Oncogenes and signal transduction*. *Cell* 1991, 64: 281-302.
97. Escobedo, J.A., Kaplan, D.R., Kavanaugh, W.M., Turck, C.W., Williams, L.T. *A phosphatidylinositol-3 kinase binds to platelet-derived growth factor receptors through a specific receptor sequence containing phosphotyrosine*. *Mol Cell Biol* 1991, 11: 1125-32.
98. Turck, C.W., Escobedo, J.A., Lewis, L.T. *Delineation of the PDGF beta-receptor sequence responsible for the association with P13-kinase using tyrosine-phosphorylated peptides*. Fifth Symposium of The Protein Society. Baltimore, MD (June 22-26) 1991, T137.
99. Sato, K., Miki, S., Tachibana, H., Hayashi, F., Akiyama, T., Fukami, Y. *A synthetic peptide corresponding to residues 137 to 157 of p60v-src inhibits tyrosine-specific protein kinases*. *Biochem Biophys Res Commun* 1990, 171: 1152-9.
100. Uehara, Y., Hori, M., Takeuchi, T., Umezawa, H. *Phenotypic change from transformed to normal induced by benzoquinonoid ansamycins accompanies inactivation of p60src in rat kidney cells infected with Rous sarcoma virus*. *Mol Cell Biol* 1986, 6: 2198-206.
101. Uehara, Y., Murakami, Y., Mizuno, S., Kawai, S. *Inhibition of transforming activity of tyrosine kinase oncogenes by herbimycin A*. *Virology* 1988, 164: 294-8.
102. Suzukaket-Suchiya, K., Moriya, Y., Hori, M., Uehara, Y., Takeuchi, T. *Induction by herbimycin-A of contact inhibition in v-Src-expressed cells*. *J Antibiot* 1989, 42: 1831-7.
103. Uehara, Y., Fukazawa, H., Murakami, Y., Mizuno, S. *Irreversible inhibition of v-src tyrosine kinase activity by herbimycin and its abrogation by sulfhydryl compounds*. *Biochem Biophys Res Commun* 1989, 163: 803-9.
104. Fukazawa, H., Mizuno, S., Uehara, Y. *Effects of herbimycin A and various SH-reagents on p60v-src kinase activity in vitro*. *Biochem Biophys Res Commun* 1990, 173: 276-82.
105. Hori, M., Uehara, Y., Takeuchi, T. *Antibiotics inhibiting oncogene functions*. In: *Antitumor Natural Products*, Vol. 36. Takeuchi, T., Nitta, K., Tanaka, N. (Eds.). Japan Scientific Societies Press: Tokyo 1989, 193-201.
106. Geissler, J.F., Traxler, P., Regenass, U., Murray, B.J., Roesel, J.L., Meyer, T., Mcglynn, E., Storni, A., Lydon, N.B. *Thiazolidine-diones biochemical and biological activity of a novel class of tyrosine protein kinase inhibitors*. *J Biol Chem* 1990, 265: 22255-61.
107. Sonoda, T., Osada, H., Uramoto, M., Uzawa, J., Isono, K. *Epiderstatin, a new inhibitor of the mitogenic activity induced by epidermal growth factor*. 2. *Structure elucidation*. *J Antibiot* 1989, 42: 1607-9.
108. Osada, H., Sonoda, T., Kusakabe, H., Isono, K. *Epiderstatin, a new inhibitor of the mitogenic activity induced by epidermal growth factor*. 1. *Taxonomy, fermentation, isolation and characterization*. *J Antibiot* 1989, 42: 1599-606.
109. Kreuter, M.H., Leake, R.E., Rinaldi, F., Muller-Klieser, W., Maidhof, A., Muller, W.E.G., Schroder, H.C. *Inhibition of intrinsic protein tyrosine kinase activity of EGF-receptor kinase complex from human breast cancer cells by the marine sponge metabolite (+)-aerophysinin-1*. *Comp Biochem Physiol* 1990, 97B: 151-8.
110. Kreuter, M.H., Bernd, A., Holzmann, H., Muller-Klieser, W., Maidhof, A., Weissmann, N., Kljajic, Z., Batel, R., Schroder, H.C., Muller, W.E.G. *Cytostatic activity of aerophysinin-1 against lymphoma and epithelioma cells*. *Z Naturforsch* 1989, 44C: 680-8.
111. Burke, T.R. Jr., Li, Z.-H., Bolen, J.B., Chapekar, M., Gang, Y., Glazer, R.I., Rice, K.C., Marquez, V.E. *Examination of the possible mediation of antineoplastic effects of opiates through the inhibition of tyrosine-specific protein kinases*. *Biochem Pharmacol* 1991, 41: R17-20.
112. Navarro, J., Abdel Ghany, M., Racker, E. *Inhibition of tyrosine protein kinases by halomethylketones*. *Biochemistry* 1982, 21: 6138-44.
113. Epand, R.M., Lester, D.S. *The role of membrane biophysical properties in the regulation of protein kinase C activity*. *Trends Pharmacol Sci* 1990, 11: 317-20.
114. Ito, S., Richert, N., Pastan, I. *Vinculin phosphorylation by the src kinase: Inhibition by chlorpromazine, imipramine and local anesthetics*. *Biochem Biophys Res Commun* 1982, 107: 670-5.
115. Igarashi, Y., Hakomori, S., Toyokuni, T., Dean, B., Fujita, S., Sugimoto, M., Ogawa, T., El-Ghendy, K., Racker, E. *Effects of chemically well-defined sphingosine and its N-methyl derivatives on protein kinase C and src kinase activities*. *Biochemistry* 1989, 28: 6796-800.
116. Steck, P.A., Hadi, A., Lotan, R., Yung, W.K.A. *Inhibition of epidermal growth factor receptor activity by retinoic acid in glioma cells*. *J Cell Biochem* 1990, 42: 83-94.
117. Literati, N.P., Keri, G., Boross, M., Nemeth, G., Szilbereky, J., Szilagyi, I. *Polyunsaturated fatty acid derivatives, pharmaceutical compositions containing the same, method for the preparation thereof, and their use as medicament*. WO 90 08, 130 (July 26, 1990), CA 114: 102847w.
118. Traxler, P.M., Wacker, O., Bach, H.L. et al. *Sulfonylbenzoyl-nitrostyrenes - Potential bisubstrate type inhibitors of the EGF-receptor tyrosine protein kinase*. *J Med Chem* 1991, 34: 2328-37.
119. Hsu, C.-Y.J., Persons, P.E., Spada, A.P., Bednar, R.A., Levitzki, A., Zilberstein, A. *Kinetic analysis of the inhibition of the epidermal growth factor receptor tyrosine kinase by lavendustin-A and its analogue*. *J Biol Chem* 1991, 266: 2105-12.